[5] Phasing on Rapidly Soaked Ions

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Introduction

There are three basic ways of solving macromolecular crystal structures: the molecular replacement method, direct methods, and the heavy-atom method. Molecular replacement involves the use of a known search model, closely similar to the macromolecule being investigated. Direct methods are routinely used to solve the structures of small molecules, where the diffraction data extend to atomic resolution. As a consequence, these two methods cannot be used to solve novel structures with crystals diffracting to lower than atomic resolution. The most general method of solving novel crystals structures is therefore a heavy-atom approach in its various modifications. In general, in this approach the initial phases are derived from differences in crystal scattering caused by the presence of a small number of heavy and/or anomalously diffracting atoms, which can be inherently present in the native macromolecule or introduced by chemical derivatization. Various types of diffraction differences can be employed. In the single or multiple isomorphous replacement (SIR or MIR) methods only the differences between the intensities of the native and derivative crystals are used. If anomalous differences are used in addition, the method becomes the isomorphous replacement with anomalous scattering (SIRAS or MIRAS) method. If only the differences between Friedel-related intensities at one or more X-ray wavelengths are used, the technique is termed single- or multiple-wavelength anomalous dispersion (SAD or MAD), respectively. The details of each of these approaches are comprehensively discussed in many classic textbooks.[1,2]

The heavy atoms, providing the initial phase information, can be present in the original macromolecule, such as certain transition metals in metalloproteins or, more recently proposed as general tools, sulfur in proteins[3,4] and phosphorus in nucleic acids.[5] However, most general is the derivatization before or after crystallization. An example of the former is

[1] T. L. Blundell and L. N. Johnson, "Protein Crystallography." Academic Press, New York, 1976.
[2] J. Drenth, "Principles of Protein X-Ray Crystallography," 2nd Ed. Springer-Verlag, Heidelberg, Germany, 1999.
[3] Z. Dauter, M. Dauter, E. de La Fortelle, G. Bricogne, and G. M. Sheldrick, *J. Mol. Biol.* **289,** 83 (1999).
[4] E. Micossi, W. N. Hunter, and G. A. Leonard, *Acta Crystallogr. D Biol. Crystallogr.* **58,** 21 (2002).
[5] Z. Dauter and D. A. Adamiak, *Acta Crystallogr. D Biol. Crystallogr.* **57,** 990 (2001).

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the production of selenomethionine protein variants[6] by genetic engineering for the MAD technique. The classic derivatization approach involves prolonged soaking of the native crystals in diluted solutions of various heavy metal salts and coordination compounds. Such soaking procedures are time consuming and often unsuccessful, owing to the lack of heavy atom binding or the deterioration of crystal quality.

Dauter and co-workers have proposed that certain simple anions or cations suitable for phasing, such as halides or alkali metals, can be introduced into protein crystals by rapid soaks in the appropriate cryo derivatization solutions[7,8] ("quick cryosoaking approach"). This procedure combines, in one rapid single step, derivatization and cryogenic protection. Immediately before freezing the crystal for data collection, a native crystal is immersed for a short period of time in a cryoprotectant solution drop containing in addition a high concentration of the appropriate salt. Compared with classic soaks, which combine low heavy-metal concentration and long immersion times, this procedure is able to generate good isomorphous derivatives significantly faster. This approach is based on somewhat different chemical behavior of halides and alkaline ions in comparison with the classic heavy-atom compounds.

Protein crystals contain a significant proportion of the liquid solvent phase, filling the voids between the more or less globular protein molecules. Various small chemical compounds can diffuse through the solvent channels within protein crystals, and this has often been used to obtain, for example, enzyme complexes with inhibitors, cofactors, and so on. This diffusion is quick, which is evidenced by the frequent presence of the glycerol molecules bound to the protein surface after a short (2- to 5-s) immersion of the crystal in the cryoprotecting solution containing glycerol.[9]

The rapid soak approach uses this property of protein crystals, which allows small ions to diffuse within a short time to the solvent regions surrounding the protein molecules and adopt ordered sites at their surface.

Ions Used for Rapid Soaks

Both negatively charged heavy halides and positively charged heavy alkali ions have been proposed for this fast derivatization approach.

[6] W. A. Hendrickson and C. M. Ogata, *Methods Enzymol.* **276,** 494 (1997).

[7] Z. Dauter, M. Dauter, and K. R. Rajashankar, *Acta Crystallogr. D Biol. Crystallogr.* **56,** 232 (2000).

[8] R. A. P. Nagem, Z. Dauter, and I. Polikarpov, *Acta Crystallogr. D Biol. Crystallogr.* **57,** 996 (2001).

[9] J. Lubkowski, Z. Dauter, F. Yang, J. Alexandratos, G. Merkel, A. M. Skalka, and A. Wlodawer, *Biochemistry* **38,** 13512 (1999).

The presence of chloride anions has been observed in the structures of proteins crystallized from solutions containing a significant concentration of sodium chloride, for example, in tetragonal lysozyme.[3,10] When lysozyme was crystallized from a solution containing NaBr[11,12] or NaI,[13] a number of sites occupied by these halides appeared at the protein surface. This observation, and the analysis of data collected on a few test crystals, led to the proposal of using soaked bromides and iodides for phasing.[7]

The two heavier halides, bromine and iodine, display a significant anomalous signal in the range of wavelength easily accessible by most synchrotron beam lines. Bromine has the K absorption edge at 0.92 Å (13,474 eV) and is appropriate for phasing by the MAD method. Bromine has one more electron than selenium and it has been used for MAD solution of oligonucleotide structures after substituting thymine by the almost isostructural bromouracil.[14] It can be considered as the nucleic acid equivalent of the SeMet substitution in protein crystallography.[6]

The iodine absorption edges (K at 0.37 Å and L_{I} at 2.39 Å) are not easily accessible, and iodine is therefore not suitable for the MAD work. However, it retains a significant anomalous signal ($f'' = 6.8$ electron units) at the copper characteristic wavelength of 1.54 Å. Iodine has been used as a heavy atom in protein crystallography after chemical modification of the tyrosine aromatic rings.[15,16]

Chlorine, the halide lighter than bromine or iodine, has its K edge at a long wavelength (4.39 Å), and displays only a small anomalous effect at more accessible wavelengths ($f'' = 0.70$ at 1.54 Å and $f'' = 0.88$ at 1.74 Å). Nevertheless, with the accurately measured data it is possible to use its anomalous signal for phasing.[3,17,18]

[10] C. C. F. Blake, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Proc. R. Soc. Lond. B Biol. Sci.* **167,** 365 (1967).

[11] K. Lim, A. Nadarajah, E. L. Forsythe, and M. L. Pusey, *Acta Crystallogr. D Biol. Crystallogr.* **53,** 240 (1998).

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[13] L. K. Steinrauf, *Acta Crystallogr. D Biol. Crystallogr.* **54,** 767 (1998).

[14] J. L. Smith and W. A. Hendrickson, *in* "International Tables for Crystallography" (M. G. Rossmann and E. Arnold, eds.), Vol. F, Chapter 14.2.1. Kluwer Academic, Dordrecht, The Netherlands, 2001, p. 299.

[15] L. Q. Chen, J. P. Rose, E. Breslow, D. Yang, W. R. Chang, W. F. Furey, M. Sax, and B. C. Wang, *Proc. Natl. Acad. Sci. USA* **88,** 4240 (1991).

[16] L. Brady, A. M. Brzozowski, Z. S. Derewenda, E. Dodson, G. Dodson, S. Tolley, J. P. Turkenburg, L. Christiansen, B. Huge-Jensen, L. Norskov, L. Thim, and U. Menge, *Nature* **343,** 767 (1990).

[17] C. Lehmann, (2000). Ph.D. thesis. University of Göttingen, Göttingen, Germany.

[18] P. J. Loll, *Acta Crystallogr. D Biol. Crystallogr.* **57,** 977 (2001).

The use of heavy alkali metals for rapid cryosoaks was proposed as an extension to the quick cryosoaking approach with halides.[8] The heavier alkali metals rubidium and cesium have two electrons more than the halides bromine and iodine, respectively. This difference, extremely important from the chemical point of view, allows Cs or Rb cations to occupy different positions in the crystal structure when compared with the positions occupied by I or Br anions. This fact adds an additional flexibility to the procedure of quick cryosoaking and permits combined use of such derivatives in MIR(AS) phasing, even when none of them are strong enough to produce interpretable electron-density maps individually.

The *K* absorption edge of rubidium (0.82 Å) is in the same range as Br and Se *K* edges, which makes it a suitable atom for MAD phasing. Indeed, it was tested as a useful MAD phasing source.[19]

On the other hand, cesium atoms, even though possessing a strong anomalous signal with $f'' = 7.90$ electrons at a wavelength of 1.54 Å, are not suitable for MAD experiments. The Cs absorption edges (*K* at 0.34 Å and L_I at 2.17 Å) are far away from the wavelength range accessible at most synchrotron beam lines. The anomalous signal of cesium has been used for phasing in the past, for example, for gramicidin.[20]

Differences between Classic Reagents and Ions Used for Quick Soaks

The ions used for the quick-soak approach differ in their chemical properties from the classic heavy atom reagents. Halides in water solution occur as not coordinated, monoatomic anions, although they interact with water through hydrogen bonds. Alkali cations are coordinated by water molecules, but the coordination is not strong, and the metal aquo ligands can be easily exchanged, for example, by the carboxyl or carbonyl oxygen atoms. In contrast, in many standard heavy-atom reagents the ligands are strongly coordinated or covalently bound to the metal.[21] To bind to the appropriate protein sites such reagents must undergo a partial hydrolysis or a similar chemical substitution. They usually form strong complexes or bind covalently to certain chemical functions of the protein, such as, for example, mercury reagents with the cysteine sulfhydryl groups. If present in higher concentration, these reagents often disrupt the protein intermolecular

[19] S. Korolev, I. Dementieva, R. Sanishvili, W. Minor, Z. Otwinowski, and A. Joachimiak, *Acta Crystallogr. D Biol. Crystallogr.* **57,** 1008 (2001).

[20] B. A. Wallace, W. A. Hendrickson, and K. Ravikumar, *Acta Crystallogr. B* **46,** 440 (1990).

[21] D. Carvin, S. A. Islam, M. J. E. Sternberg, and T. E. Blundell, *in* "International Tables for Crystallography" (M. G. Rossmann and E. Arnold, eds.), Vol. F, Chapter 12.1. Kluwer Academic, Dordrecht, The Netherlands, 2001, p. 247.

interactions, damaging the crystalline order and adversely influencing the crystal diffraction. The usual procedures involve long (several hours or days) soaks at low, millimolar concentration of the appropriate reagent, allowing the chemical reactions to proceed slowly.

Bromide and iodide anions are soft, monoatomic, and polarizable. They are attracted to the protein surface through various types of relatively weak, noncovalent interactions. First, because of their negative charge they can form ion pairs with the positively charged arginine and lysine side chain functions. Second, they can accept hydrogen bonds from various proton donors, such as protein amides (in the main or side chains) and hydroxyls, as well as solvent water molecules. Third, they can interact with the protein hydrophobic surfaces. All these interactions are observed in protein crystals containing halide sites. With respect to chemical interactions with proteins, halides are not highly specific. The halide-bonding interactions do not require any slow chemical reaction to take place and can be formed quickly.

The cations are more specific in the character of their binding. Alkali ions have a preference for oxygen functions such as carboxyls (negatively charged), carbonyls, or water. Their sites are in the vicinity of a few side-chain carboxyls, or main- and side-chain carbonyls, available at the protein surface, and usually have a number of water ligands. Rubidium and cesium ions are not strongly demanding of the coordination geometry and can have five to eight ligands.[19] Because they do not coordinate water molecules strongly, the substitution of water ligands by the protein oxygens takes place rapidly.

As stated above, binding of halides and alkali ions is not strong, and their sites around the protein surface are partially occupied even if their concentration in the mother liquor is higher than 1 *M*. All these ions can share the sites with water molecules, and their occupancy results from the competition between them and water in binding to various protein functions with variable strength in the state of equilibrium. It is difficult to estimate accurately the absolute occupancy factors of these sites. The relative occupancies of the strongest anomalous sites in a few example structures are given in Table I.

Figure 1 illustrates the most typical sites and coordination of soaked ions from the crystal structures solved by their use.

Procedure

A number of macromolecular structures from a variety of organisms with different functions have been solved by using the quick cryosoaking approach with halides and alkaline metals. In Table II[8,22–34] we show several derivatization aspects of some of these structures, including size of protein,

soaking time, cryoderivatization conditions, and so on. It is easy to see from Table II that there is not a general recipe for all types of proteins. However, a few instructive steps, acquired with practice, could be followed to enhance the chance of obtaining suitable derivatives for phasing.

Normally, the cryoderivatization solution is prepared from the original mother liquor. The simple addition of a cryoprotectant and an appropriate salt in high concentration is often enough to produce a good cryoderivatization solution. Depending on the crystallization conditions, a complete or partial substitution of salts containing various anions by bromides or iodides also can be performed. As in the SptP:SicP complex structure determination,[33] the sodium chloride used during crystallization was completely replaced by sodium bromide during derivatization. Analogously, lithium, sodium, and potassium can be replaced by cesium or rubidium. In cases with a saturated crystallization solution, a partial substitution of reagents is likely to work.

The results indicate that the quick cryosoaking approach can be used with success under a number of adverse crystallization conditions. Different types of precipitants have been used so far, including several polyethylene glycol (PEG) sizes, ammonium sulfate in various concentration, and others. The use of other additives as detergent,[26] azide,[25] sucrose,[27] and even ammonium sulfate in high concentration[30] seems not to affect the derivatization drastically.

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[31] L.-J. Baker, J. A. Dorocke, R. A. Harris, and D. E. Timm, *Structure* **9,** 539 (2001).

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[33] C. E. Stebbins and J. E. Galán, *Nature* **414,** 77 (2001).

[34] A. Rojas, R. A. P. Nagem, K. N. Neustroev, A. M. Golubev, E.V. Eneyska, A. A. Kulminskaya, and I. Polikarpov, in preparation (2003).

TABLE I

ANOMALOUS SCATTERER SITES IN SOME CRYSTAL STRUCTURES[a]

β-Galactosidase (iodine)						β-Galactosidase (cesium)					
	Fractional coordinates			*Peak height*			*Fractional coordinates*			*Peak height*	
Site	*x*	*y*	*z*	*σ*	*Normative*	*Site*	*x*	*y*	*z*	*σ*	*Normative*
I-1	0.0405	0.3618	0.7993	31.31	1.00	Cs-1	0.7358	0.5808	0.4773	31.06	1.00
I-2	0.1530	0.1618	0.8228	30.81	0.98	Cs-2	0.8417	0.5499	0.4459	25.31	0.81
I-3	0.4482	0.1546	0.7447	19.90	0.64	Cs-3	0.9259	0.5141	0.7811	23.20	0.75
I-4	0.4558	0.0530	0.8035	19.21	0.61	Cs-4	0.4873	0.5272	0.6456	20.40	0.66
I-5	0.1338	0.4844	0.9018	14.92	0.48	Cs-5	0.7780	0.3222	0.7513	9.67	0.31
I-6	0.0706	0.4947	0.6388	14.73	0.47	Cs-6	0.9676	0.3061	0.7619	7.95	0.26
I-7	0.3282	0.3609	0.9262	14.24	0.45	Cs-7	0.5367	0.1733	0.4704	7.88	0.25
I-8	0.4323	0.4568	0.9342	13.94	0.45	Cs-8	0.8344	0.5700	0.6374	6.53	0.21
I-9	0.4217	0.5679	0.9902	13.57	0.43	Cs-9	0.5718	0.3767	0.6471	5.81	0.19
I-10	0.5587	0.0003	0.9921	12.77	0.41	Cs-10	0.0482	0.2172	0.6737	5.39	0.17

Acyl protein thioesterase I (bromine)						Trypsin inhibitor (cesium)					
	Fractional coordinates			*Peak height*			*Fractional coordinates*			*Peak height*	
Site	*x*	*y*	*z*	*σ*	*Normative*	*Site*	*x*	*y*	*z*	*σ*	*Normative*
Br-1	0.0724	0.2361	0.6181	41.92	1.00	Cs-1	0.4544	0.1594	0.3748	25.22	1.00
Br-2	0.3298	0.1346	0.8744	41.50	0.99	Cs-2	0.6969	0.1170	0.2326	21.74	0.86
Br-3	0.9559	0.9969	0.1666	30.58	0.73	Cs-3	0.7012	0.2988	0.2500	20.00	0.79
Br-4	0.7732	0.3760	0.0022	24.19	0.58	Cs-4	0.5693	0.4503	0.3392	18.00	0.71
Br-5	0.1386	0.1800	0.6227	19.59	0.47	Cs-5	0.1248	0.5987	0.3385	17.46	0.69
Br-6	0.8183	0.0550	0.3299	19.17	0.46	Cs-6	0.4357	0.5200	0.3344	15.53	0.62
Br-7	0.6100	0.3168	0.1365	18.81	0.45	Cs-7	0.5996	0.3860	0.1891	12.87	0.51
Br-8	0.3252	0.1924	0.8098	18.35	0.44	Cs-8	0.0984	0.4384	0.2413	10.35	0.41
Br-9	0.8060	0.3409	0.4412	17.33	0.41	Cs-9	0.6120	0.1409	0.1027	10.15	0.40
Br-10	0.4539	0.1646	0.3777	16.59	0.40	Cs-10	0.3589	0.5542	0.2645	7.56	0.30

[a] Described in Table II. The 10 strongest sites are listed with their corresponding peak heights given in σ and normalized to the highest peak in the anomalous difference-Fourier map.

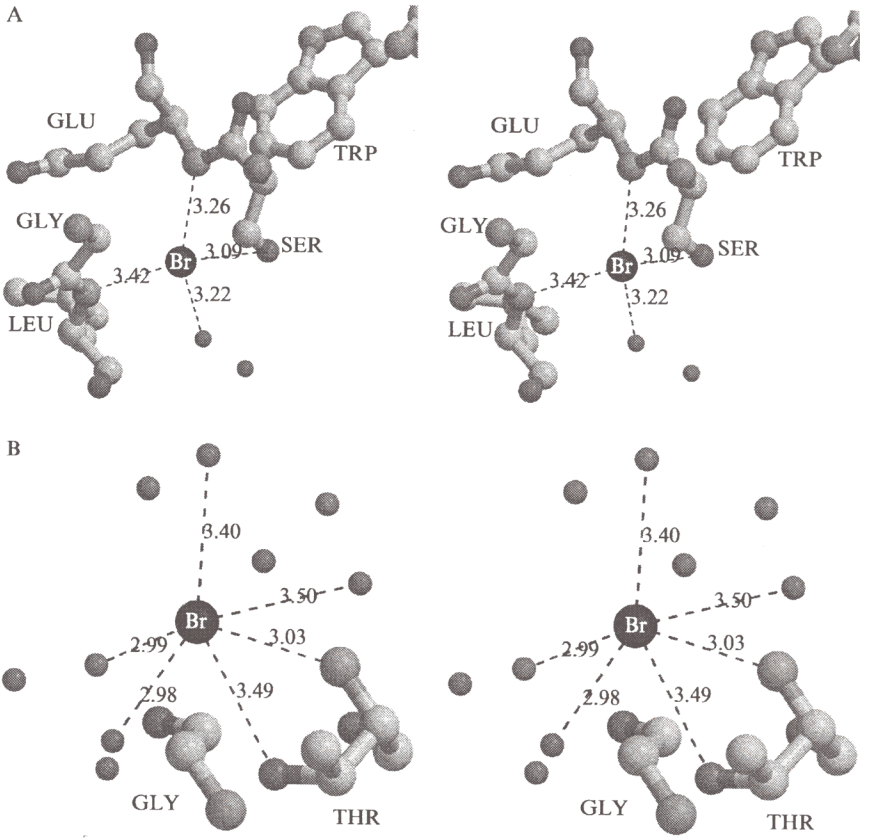

FIG. 1. (*Continues*)

The choice between ethylene glycol and glycerol for cryogenic protection is in principle not too relevant for the derivatization process itself. It should rather be chosen for each solution in order to provide complete cryogenic protection.

Another decision that must be made before preparation of the derivative is the choice of the correct salt. If MAD data collection can be performed, the use of bromide and rubidium salts such as NaBr, KBr, or RbCl is recommended. The pH of the mother liquor may suggest the most appropriate salt. In principle, in low pH the protein molecules are positively charged, which therefore makes halides a better option. At high pH the alkaline metals may be recommended. These recommendations are based more on chemical intuition than experience, and more extensive

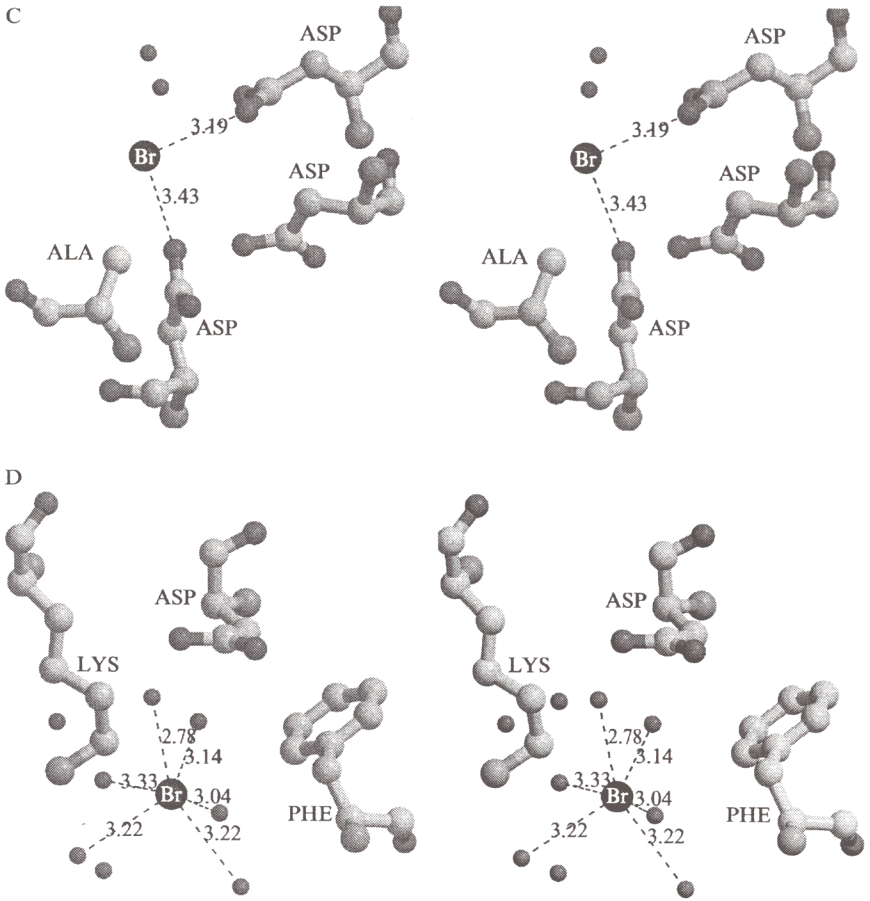

FIG. 1. (*Continued*)

studies with different pH values are required for a final conclusion. On the other hand, if the MAD approach is not applicable, the use of iodide (LiI, NaI, or KI) or cesium (CsCl) salts combined with a longer X-ray wavelength is advised.

It seems appropriate to mention that even though the content of the asymmetric unit can only be estimated during data collection, and such information cannot be used to verify the applicability of the quick cryo-soaking approach, the results indicate that even larger macromolecules such as β-galactosidase[34] or the SptP:SicP complex[33] can be solved by this technique. Similarly, macromolecular crystals with a solvent content as low as 35% have already been solved.[26,24]

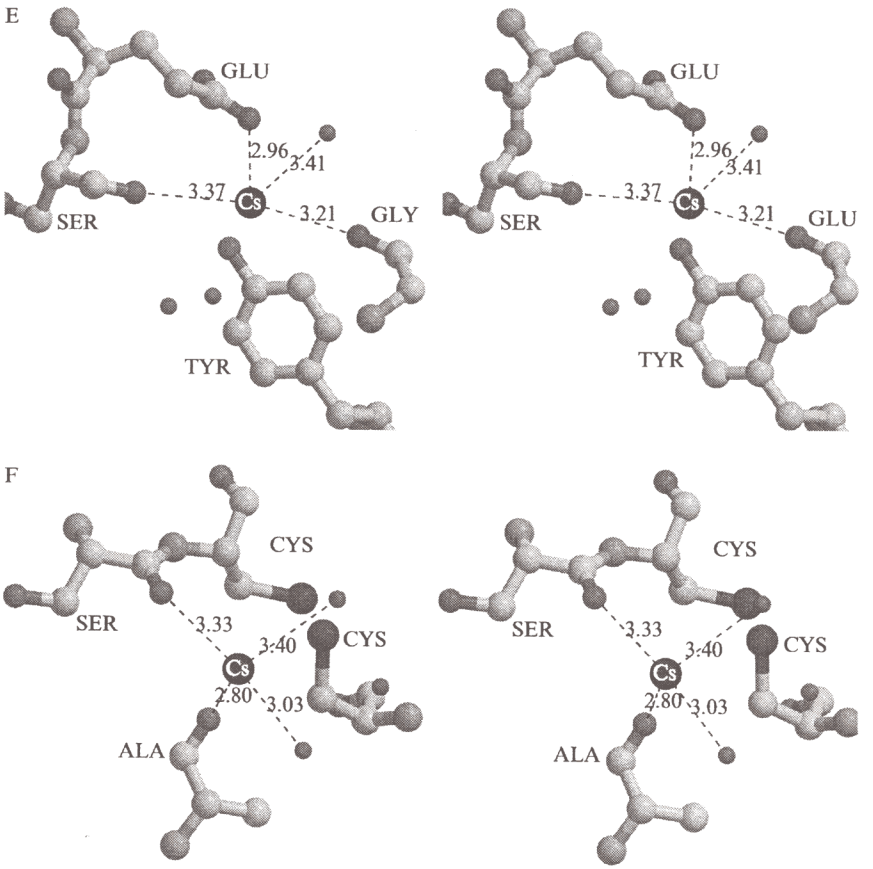

FIG. 1. (*Continued*)

Bromides and iodides do not require long soaking times. Experience shows that soaking for longer than 30 s is not necessary, and may lead to deterioration of the diffraction power. Surprisingly, it has been observed that short halide soaks can improve the crystal diffraction[35] and sometimes even cause the crystal phase transition to a different symmetry.[36] Metal ions seem to require somewhat longer soaking for successful derivatization.

[35] M. Harel, R. Kasher, A. Nicolas, J. M. Guss, M. Balass, M. Fridkin, A. B. Smit, K. Brejc, T. K. Sixma, E. Katchalski-Katzir, J. L. Sussman and S. Fuchs, *Neuron* **32,** 265 (2001).
[36] Z. Dauter, M. Li, and A. Wlodawer, *Acta Crystallogr. D Biol. Crystallogr.* **57,** 239 (2001).

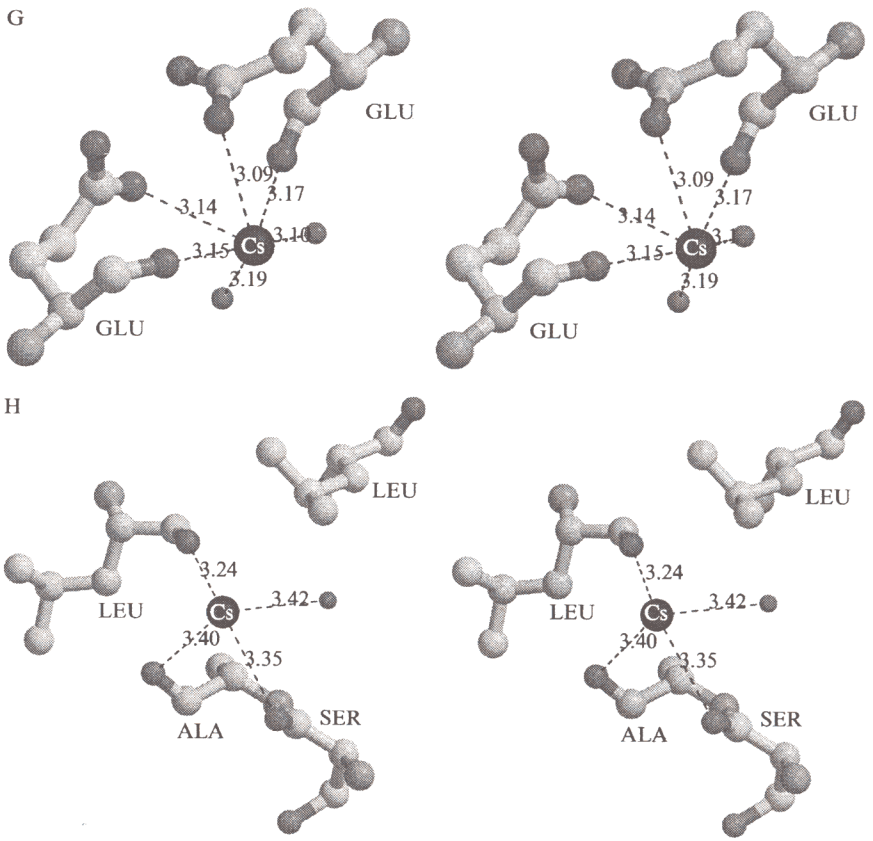

FIG. 1. Stereo plot of the representative sites of cryosoaked ions. Surrounding residues are shown if they have at least one atom closer than 4.5 Å from the ion. Coordination distances to polar atoms closer than 3.5 Å are marked. (A–D) Bromide sites in acyl protein thioesterase I; (E–H) cesium sites in trypsin inhibitor.

Examples

α-*Galactosidase from* Trichoderma reesei

The first crystals of α-galactosidase from *Trichoderma reesei* were obtained in 1993.[37] Since then, much effort has been spent on solving the phase problem for this protein. In spite of using a number of heavy metals for derivatization (nearly 20 chemicals including Pt, Ag, Au, U, W, and

[37] A. M. Golubev and K. N. Neustroev, *J. Mol. Biol.* **231,** 933 (1993).

TABLE II
EXAMPLES OF CRYSTAL STRUCTURES SOLVED BY CRYOSOAKING

Protein	Size (au) kDa	Solvent content (%)	Cryoderivatization conditions	Soak time (s)	Phasing method	No. of sites	Resolution (Å)[a]	Ref.
Insulin-like growth factor-I from *Homo sapiens*	1 × 6	55	25% (w/v) PEG 3350, 30% MPD, 0.2 *M* sodium cacodylate (pH 6.5), 2.8 m*M* deoxy-BIGCHAP, 1.0 *M* NaBr	30	MAD	1 Br plus 6 Cys Sγ	2.00 (1.80)	22
β-Defensin-2 from *Homo sapiens*	4 × 4	40	36% PEG 4000, 0.32 *M* lithium sulfate, 0.16 *M* MOPS (pH 7.1), 10% glycerol, 0.25 *M* KBr (0.25 *M* Kl)	60	MIRAS	9 Br (9 I)	2.00 (1.40)	23
C-terminal domain of TonB from *Escherichia coli*	2 × 8	35	28–30% PEG 3350, 0.1 *M* Tris (pH 7.5), 50–100 m*M* calcium chloride, 1.0 *M* KBr	50	MAD	4 Br	2.50 (1.55)	24
Peroxiredoxin 5 from *Homo sapiens*	1 × 17	65	1.6 *M* ammonium sulfate, 0.1 *M* sodium citrate (pH 5.3), 0.2 *M* potassium sodium tartrate, 1 m*M* 1,4-dithio-DL-threitol, 0.02% (w/v) azide, 20% (v/v) glycerol, 1.0 *M* NaBr	30	MAD	5 Br	1.90 (1.50)	25
Trypsin inhibitor from *Copaifera langsdorffi*	1 × 18	45	20–25% PEG 8000, 0.1 *M* sodium acetate (pH 4.5), 20% ethylene glycol, 1.0 *M* CsCl	300	SIRAS	5 Cs	2.00 (2.00)	8
Interleukin-22 from *Homo sapiens*	2 × 17	33	0.9 *M* sodium tartrate, 0.1 *M* HEPES (pH 7.5), Triton X-100 detergent, 15% ethylene glycol, 0.125 *M* Nal	180	SIRAS	10 I	1.92 (1.92)	26
GAF domain YKG9 from *Saccharomyces cerevisiae*	2 × 18	65	2.5 *M* ammonium sulfate, 0.05 *M* lithium sulfate, 30% sucrose, 0.1 *M* Tris-HCl (pH 8.0), 10% (v/v) glycerol, 0.5 *M* NaBr	45	MAD	7 Br	2.80 (1.90)	27

Carboxyl proteinase from *Pseudomonas* sp. 101	1 × 41	56	1.0 *M* ammonium sulfate, 0.005 *M* guanidine, 0.1 *M* sodium citrate (pH 3.3),18% glycerol, 1.0 *M* NaBr	30	SAD	9 Br	1.80 (1.40)	28
α-Galactosidase from *Trichoderma reesei*	1 × 47	47	15% PEG 3350, 100 m*M* potassium phosphate, 10% glycerol, 0.26 *M* CsCl	480	SIRAS	10 Cs	1.60 (1.60)	29
Acyl protein thioesterase I from *Homo sapiens*	2 × 25	38	42% saturation ammonium sulfate, 0.1 *M* sodium acetate (pH 5.0), 20% (v/v) glycerol, 1.0 *M* NaBr	20	SAD	22 Br	1.80 (1.50)	30
Thiamine pyrophosphokinase from *Saccharomyces cerevisiae*	2 × 35	49	25% PEG-MME 2000, 0.1 *M* ammonium sulfate, 0.1 *M* sodium acetate (pH 5.1), 50 m*M* sodium chloride, 1.0 *M* NaBr	45	MAD	12 Br	2.00 (1.80)	31
Cysteine-rich domain of Sfrp-3 from *Mus musculus*	6 × 14	45	0.1 *M* HEPES (pH 6.6), 33% PEG 3350, 0.5 *M* NaBr	40	MAD	9 Br	1.90 (1.90)	32
SptP:SicP complex (2:4) from *Salmonella typhimurium*	2 × 18 4 × 12	58	5–10% PEG 6000, 15% glycerol, 2.0 *M* NaBr	30	MAD	31 Br	2.50 (1.90)	33
β-Galactosidase from *Penicillium* sp.	1 × 110	72	15% PEG 8000, 50 m*M* sodium phosphate (pH 4.0), 30% ethylene glycol, 0.25 *M* NaI (CsCl)	180 (300)	SIRAS	13 I (12 Cs)	1.95 (1.85) 2.04 (1.85)	34

[a] Data set resolution used for phasing. Resolution in parentheses refers to the maximum resolution of all data sets used.

rare earth elements), all "derivatives" suffered the absence of heavy-atom binding.

The method of quick cryosoaking was then used to overcome this difficulty. In the first trial, native crystals of α-galactosidase ($P2_12_12_1$; $a = 46.5$, $b = 79.1$, $c = 119.4$ Å) were soaked in a cryoprotectant solution containing in addition 0.1–0.5 *M* KI and then used for X-ray diffraction data collection. Initially, these diffraction data, collected at the PCr beamline[38] at the Brazilian National Synchrotron Light Source (LNLS), could not be used for phasing because the search for iodine binding sites failed (nonisomorphism was also observed; $a = 41.9$, $b = 79.9$, $c = 120.0$ Å).

A second quick derivative, prepared with 0.2 *M* CsCl in the cryoprotectant solution, was then used for 1.6-Å resolution data collection at the same beamline. The incorporation of Cs atoms was successful and RSPS[39] and SnB[40] programs found a few equivalent Cs sites independently, using solely the anomalous signal of Cs atoms. However, similarly to the I-soaked crystal, the Cs-soaked crystal ($a = 42.1$, $b = 80.4$, $c = 120.0$ Å) was nonisomorphic to the native. Both soaked crystals showed a difference of almost 10% in the a cell parameter compared with the native crystals. Therefore, data for the iodine pseudo-derivative and the Cs derivative of α-galactosidase were used as native and derivative, respectively, for initial SIRAS phasing. Ten Cs sites were used by SHARP[41] in the SIRAS approach, followed by DM,[42] and the resulting phases were good enough for automatic model building by wARP.[43]

β-*Galactosidase from* Penicillium *Species*

One of the highest molecular weight protein structures solved so far with a derivative prepared according to the quick cryosoaking procedure was β-galactosidase from a *Penicillium* sp., with 110 kDa (one molecule) in the asymmetric unit. Initial X-ray diffraction studies revealed that β-galactosidase crystallized in space group $P4_3$ with unit cell parameters $a = b = 110.9$ Å, $c = 161.0$ Å and diffracted to 1.85-Å resolution.

An iodine derivative was prepared by immersion of a native crystal in mother liquor solution containing, in addition, 0.25 *M* NaI and 30% ethylene glycol. Crystals were visually stable in the derivatization solution and

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did not suffer large changes in cell parameters or loss of diffraction power compared with native crystals. The SnB[40] program used the normalized anomalous differences of this derivative to locate the halide substructure. Phase calculation performed by SHARP[41] in the SIRAS approach with 13 iodine sites gave a mean figure of merit of 0.37 in the 27.0- to 2.60-Å resolution range. The final electron density map obtained after density modification with SOLOMON[44] was used by wARP[43] for automatic model building. The number of built residues was increasing in each cycle; however, the convergence was slow and 3 days were required to obtain the final model (95% complete).

Even though just one halide derivative was used for phasing and solving of the crystal structure, we mention that a second quick cryo soaked derivative was obtained during model building. At this time, CsCl was used instead of NaI in the derivatization solution. Twelve cesium sites were used for SIRAS phasing. Similar to the first phase calculation, this one gave a mean figure of merit of 0.37 in the same resolution range. When native and both derivative data sets were combined in MIRAS phasing, the resulting figure of merit was 0.52 and the electron density map showed significant improvement compared with either of the SIRAS maps.

Acyl Protein Thioesterase

Crystals of human acyl protein thioesterase I were grown from a solution containing a high concentration of ammonium sulfate in a monoclinic cell with two molecules of 228 residues each in the asymmetric unit. They were soaked for 20 s in the mother liquor with added 1 *M* NaBr. The diffraction data were collected to 1.8-Å resolution at an energy 50 eV higher than the Br absorption edge. The data displayed a clear anomalous signal and the structure was solved by SAD, using only this data set.

The initial seven Br sites were located by SnB.[40] They were input to SHARP,[41] which after two iterations identified 22 Br sites in the residual maps and produced a phase set with an overall figure of merit of 0.40. The strongest 18 Br sites formed two groups of almost identical constellations, clearly identifying the presence of a noncrystallographic 2-fold axis. The application of density modification with DM[42] increased the figure of merit to 0.85 and produced an easily interpretable map. The majority of the residues were built automatically using wARP.[43] At the end of the refinement, the anomalous difference Fourier map identified a total of 40 bromide sites located at the surface of the two independent protein molecules. They were included in the refinement of the final model with either full

[44] J. P. Abrahams and A. G. W. Leslie, *Acta Crystallogr. D Biol. Crystallogr.* **52,** 30 (1996).

or half occupancies, depending on the appearance of the corresponding peaks in the anomalous difference map.

Conclusions

The quick cryosoaking approach was established in 2000, and since then a number of macromolecular crystal structures have been solved with this method. The results obtained so far indicate that, owing to its several intrinsic aspects, it can be particularly applicable for high-throughput crystallographic projects.

This approach can be used with a great number of different crystallization solutions, and the presence of various compounds, such as sugars, additives, or even precipitants, in high concentration does not impede the fast incorporation of halide or alkaline metal ions to the solvent regions surrounding the protein molecules. Moreover, halides or alkaline metals are less likely to react with certain compounds that are used during crystallization than are some heavy-metal salts (e.g., they do not precipitate with phosphate anions).

Another interesting point is that little preparative effort and only a short time are required to produce a potential derivative. All the equipment and chemical compounds used in this approach can be found in a simple protein crystallization laboratory. A single 2- to 5-μl derivatization solution drop is used in each trial and the crystal soak time is usually less than 1 min. In addition, one can see immediately whether the crystal is stable or not in solution and can modify the salt concentration and/or soaking time to obtain a better derivative.

The choice between a halide and an alkaline metal salt must be made before the derivatization procedure; this selection does not mean that a second salt cannot also be used for another derivative. Sometimes this decision can be made easily, depending on the types of compounds used in the crystallization solution. Iodides or bromides can replace chlorides. Similarly, lithium, sodium, and potassium can be substituted by cesium or rubidium. Because preparation of derivatives is fast, and data collection normally is performed with frozen crystals, both types of derivatives can be prepared and immediately frozen for data collection. Moreover, the use of halide and alkaline metal salts during derivatization opens up the possibility of using two essentially different derivatives to solve a protein structure through MIR(AS) when none of them alone is able to do so.

Bromide and rubidium salts have an advantage over iodide and cesium salts, in that the former can be easily used for MAD because their *K* absorption edges are in the similar energy range as the Se edge, the scatterer used most often for MAD phasing. The latter derivatives, on the other hand, with *K* absorption edges in the vicinity of 0.35 Å, are not suitable

for MAD phasing, even though some X-ray diffraction experiments have been done at this energy.[45] Nevertheless, iodine and cesium atoms possess significant anomalous signal at longer wavelengths that makes them appropriate for SIRAS, MIRAS, and SAD phasing. Specifically at the copper-anode characteristic wavelength (1.54 Å), f'' of I and Cs atoms are 6.8 and 7.9 electrons, respectively.

In general this new approach was proposed as an alternative way of preparing derivatives when a protein does not bind heavy-metal atoms or is not amenable to the preparation of a SeMet variant.

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[6] The Bijvoet-Difference Fourier Synthesis

By JEFFREY ROACH

Introduction

The is no dispute over the profound influence anomalous dispersion-based techniques have had on macromolecular X-ray crystallography. Anomalous difference Patterson synthesis and both single and multiple anomalous dispersion phasing have become standard techniques of structural analysis. Typically the initial goal of such an analysis is the determination of the locations of the anomalously scattering atoms. Bijvoet-difference Fourier synthesis provides both a method of establishing absolute configuration and an approximation to the heavy atom substructure of the system in question. In general, this approximation is sufficient to identify the location of the most significant anomalous scatterers. Once these locations are known, the positions of the minor anomalous scatterers can be determined by the more delicate imaginary Fourier synthesis.

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